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In vitro production of infectious *Plasmodium falciparum* sporozoites

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An effective vaccine is needed for the prevention and elimination of malaria. The only immunogens that have been shown to have a protective efficacy of more than 90% against human malaria are *Plasmodium falciparum* (*Pf*) sporozoites (PfSPZ) manufactured in mosquitoes (mPfSPZ)[1–7]. The ability to produce PfSPZ in vitro (iPfSPZ) without mosquitoes would substantially enhance the production of PfSPZ vaccines and mosquito-stage malaria research, but this ability is lacking. Here we report the production of hundreds of millions of iPfSPZ. iPfSPZ invaded human hepatocytes in culture and developed to mature liver-stage schizonts expressing *P. falciparum* merozoite surface protein 1 (PfMSP1) in numbers comparable to mPfSPZ. When injected into FRGhuHep mice containing humanized livers, iPfSPZ invaded the human hepatocytes and developed to PfMSP1-expressing late liver stage parasites at 45% the quantity of cryopreserved mPfSPZ. Human blood from FRGhuHep mice infected with iPfSPZ produced asexual and sexual erythrocytic-stage parasites in culture, and gametocytes developed to PfSPZ when fed to mosquitoes, completing the *P. falciparum* life cycle from infectious gametocyte to infectious gametocyte without mosquitoes or primates.

Culturing in vitro the asexual erythrocytic stages of *P. falciparum* revolutionized malaria research[8,9]. It was next established that sexual erythrocytic stages could be cultured[10] that were infectious to mosquitoes[11]. In 1993, PfSPZ were produced in vitro without mosquitoes[12] but in minimal quantities and they were not shown to be infectious. To our knowledge, there have been no subsequent reports.

From 2000 to 2014, malaria cases and deaths were reduced significantly by integrated control measures[13]. Despite an annual investment of US$3–4 billion, since 2015, there has been an increase in deaths caused by malaria; new tools are needed[13]. The most cost-efficient means of controlling any infectious disease is vaccination. A PfSPZ vaccine, composed of radiation-attenuated, aseptic, purified, cryopreserved PfSPZ has been or is being assessed in more than 2,000 participants aged 5 months to 61 years in 6 African countries, Europe, Indonesia and the USA[2,3,5,14–17]. The chemo-attenuated vaccine PfSPZ-CVac[4] provides 100% protection at 3 months against controlled human malaria infection with a highly variant *P. falciparum* parasite[6]. Genetically attenuated PfSPZ vaccines have entered clinical development, with attention focused on PfSPZ that arrest late in the liver stage[18].

All PfSPZ-based vaccines and PfSPZ for controlled human malaria infection are produced in aseptic mosquitoes[1]. Development of an in vitro method for the production of PfSPZ without mosquitoes would transform the manufacture of PfSPZ, substantially reduce the cost of goods and revolutionize *P. falciparum* mosquito-stage research.

Production of oocysts and iPfSPZ

Initial experiments were based in part on a hybrid of methods for in vitro culture of *Plasmodium gallinaceum*, *P. falciparum, Plasmodium berghei* and *Plasmodium yoelii* SPZ[12,19–21] (Supplementary Table 1). Stage V *P. falciparum* gametocytes were processed to produce gametes and zygotes, and then pipetted into eight-well chamber slides previously coated with Matrigel as a matrix and seeded with *Drosophila melanogaster* Schneider (S2) cells as feeder cells. Zygotes transformed to retort forms and then motile ookinetes that invaded the Matrigel and developed to oocysts (Extended Data Fig. 1a–c). Three-day-old oocysts expressed the *P. falciparum* sexual-stage antigen Pfs25[22] and 8-day-old oocysts expressed *P. falciparum* circumsporozoite protein (CSP)[23], as do mosquito-produced oocysts, and were similar in size to

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mosquito-produced 3- and 8-day-old oocysts (Extended Data Fig. 1c,d). By day 23 after culture initiation, very few iPfSPZ had been released into the medium. Thus, oocysts were dissociated mechanically and numerous iPfSPZ were observed (Extended Data Table 1).

At Sanaria, when fed through a synthetic membrane, each mosquito ingests about 5 µl blood containing typically approximately 22,000 stage V gametocytes, around 0.22% of which convert to oocysts. The in vitro transformation of stage V gametocytes to 3- and 8-day-old oocysts was substantially more efficient than in mosquitoes (Extended Data Table 2). The conversion rate from oocysts to iPfSPZ was lower than in mosquitoes, but overall conversion from stage V gametocytes to iPfSPZ was higher than in mosquitoes (Extended Data Table 1).

Simplification of production

We first assessed the need for S2 cells. Using gametocytes from the same cultures, as compared to wells with S2 cells, those without S2 cells produced similarly sized but far fewer 8-day-old oocysts (Supplementary Table 2) containing greatly reduced numbers of iPfSPZ (Extended Data Fig. 1f,g). Sf21 cells from *Spodoptera frugiperda* were comparable to S2 cells in supporting the development of iPfSPZ (Extended Data Fig. 1e).

Matrigel is produced from rat sarcoma cells and is therefore not well suited for good manufacturing practices (GMPs)[24]. To simplify and expand the size of cultures, we moved from the eight-well chamber slides to twelve-well plates and, as a substitute for Matrigel, added collagen type I from rat tails or GMP-grade collagen type I from cows. The rat tail (Fig. 1a) and bovine collagen type I supported about 58% and 56% production of iPfSPZ compared with Matrigel (Extended Data Fig. 1e). However, when partially purified iPfSPZ made with GMP-grade collagen were injected into humanized mice, all of the mice died 1–2-days after injection, possibly owing to collagen particulate matter present in the iPfSPZ preparations.

Gamete/zygote preparations added to 12-well culture plates with S2 feeder cells but without Matrigel or collagen developed into oocysts, but at a 2.6-fold lower conversion rate compared with in cultures with Matrigel and at a 2.8-fold higher rate than in mosquitoes (Extended Data Table 1). We conducted all of the subsequent experiments using this matrix-free approach and produced millions of iPfSPZ in multiple experiments (Extended Data Table 3).

iPfSPZ develop in hepatocyte cultures

The infectivity of iPfSPZ (produced in 12-well plates without matrices) to hepatocytes in culture was determined by assessing the numbers of 6-day-old liver-stage parasites with a diameter of ≥10 µm and expressing *P. falciparum* merozoite surface protein 1 (PfMSP1) within infected HC-04 cells (Fig. 1b) or primary human hepatocytes (PHHs) (Fig. 1f). iPfSPZ (10 assays) or mPfSPZ (7 assays), at 5×10^4 PfSPZ per well, were added to HC-04 cells and, 6 days later, there was a median of 58 and 36 liver stage parasites per well expressing PfMSP1, respectively (Fig. 1c). Liver-stage schizonts were 15.1 ± 5.2 µm ($n = 20$) and 14.0 ± 3.9 µm ($n = 20$) in diameter in two experiments with iPfSPZ and 15.7 ± 7.3 µm ($n = 20$) with mPfSPZ ($P > 0.05$). Similar results were observed in cultures to which Matrigel or collagen had been added (Extended Data Tables 4 and 5). iPfSPZ produced in 12-well plates without collagen invaded and developed in PHHs, and expressed *P. falciparum* liver-stage antigen 1 (PfLSA1), *P. falciparum* exported protein 1 (PfEXP1) and PfMSP1 (Fig. 1d–f), which are known to be expressed later in the liver stage. In PHHs, the numbers of 6-day-old parasites with a diameter of ≥10 µm and expressing PfHSP70, PfLSA1 or PfMSP1 were higher in cultures from iPfSPZ compared with mPfSPZ (Supplementary Table 3). In a single experiment in HC-04 cells (triplicate technical replicates), iPfSPZ (fresh and cryopreserved) from the supernatant of a hollow fibre cartridge (HFC) culture produced more late liver-stage parasites expressing PfMSP1 than mPfSPZ (fresh and cryopreserved)/(Fig. 1g).

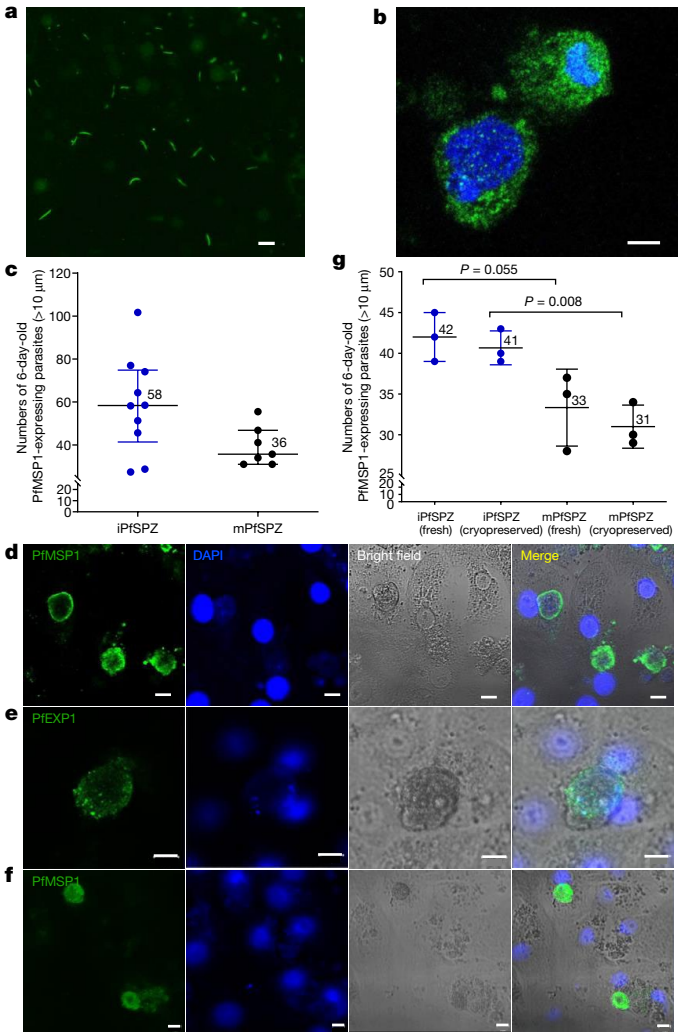

Fig. 1 | Late liver stage parasites 6 days after adding 5×10^4 iPfSPZ or mPfSPZ to triplicate wells of HC-04 cells or PHHs. a, iPfSPZ from dissociated oocysts expressing PfCSP produced in 12-well culture plates with rat-tail-derived collagen I. Scale bar, 10 µm. **b**, Confocal micrograph of 6-day-old liver-stage schizonts in HC-04 cells after infection with iPfSPZ and detected by immunofluorescence assay (IFA) using anti-PfMSP1 monoclonal antibodies and the nuclear dye DAPI. Scale bar, 5 µm. **c**, Each point is the mean number of PfMSP1-expressing parasites >10 µm in diameter in triplicate wells of HC-04 cells after incubation of iPfSPZ or mPfSPZ for 6 days. Data are median ± interquartile range (IQR) of schizonts from 10 independent experiments with iPfSPZ and 7 with mPfSPZ; the increased levels with iPfSPZ did not reach the level of statistical significance ($P = 0.084$, Mann–Whitney *U*-test). **d**–**f**, Confocal micrographs of 6-day-old liver-stage parasites in PHHs after infection with iPfSPZ and detected by IFA using anti-PfLSA1 polyclonal serum (**d**), anti-PfEXP1 monoclonal antibodies (**e**) or anti-PfMSP1 monoclonal antibodies (**f**), demonstrating that iPfSPZ invaded and developed in PHHs. For **d**–**f**, scale bars, 10 µm. **g**, The numbers of PfMSP1-expressing parasites >10 µm in diameter in triplicate wells of HC-04 cells after incubation of fresh and cryopreserved iPfSPZ or mPfSPZ for 6 days. Data are median ± IQR of schizonts from triplicate wells; the numbers of parasites expressing PfMSP1 were greater with fresh and cryopreserved iPfSPZ than with mPfSPZ.

iPfSPZ develop in humanized mouse livers

Partially purified iPfSPZ produced in 12-well plates without matrices or purified, cryopreserved mPfSPZ were injected intravenously into mice containing humanized livers (FRGhuHep). Then, 6 and 7 days later, the mice received transfusions of human erythrocytes and, 7 h after the last transfusion, blood from the FRGhuHep mice was put into *P. falciparum*

culture. Asexual and sexual *P. falciparum* erythrocytic-stage parasites were detected in two consecutive experiments. The development of blood-stage infections from iPfSPZ was slower than from mPfSPZ (Table 1); asexual *P. falciparum* were detected by blood smear on day 7 after injection in FRGhuHep mice receiving mPfSPZ, in contrast to after 19 days in culture from mice injected with iPfSPZ. This delay was not a consequence of a reduced blood-stage replication rate as, once parasites reached a detectable level, the growth rates in culture were similar whether derived from iPfSPZ or mPfSPZ (data not shown). This suggested a substantial reduction in the numbers of parasites released into the bloodstream. We therefore examined using immunofluorescence and confocal microscopy liver sections from the FRGhuHep mice infected 7 days previously, and estimated the numbers of parasites with a diameter of 10–20 µm expressing PfLSA1 (early-to-late liver stages) and schizonts with a diameter of ≥30 µm expressing PfMSP1 (late liver stages) (Table 2). Schizonts in the livers of FRGhuHep mice from iPfSPZ and mPfSPZ were of similar sizes (Fig. 2). In two experiments, the densities of early-to-late liver-stage parasites in liver sections from iPfSPZ were 68% and 38% compared with mPfSPZ, and for late liver-stage schizonts, 61% and 29% compared with mPfSPZ, respectively (Table 2). The intensity of PfLSA1 expression, which is first seen early in the liver stage, was similar in iPfSPZ- and mPfSPZ-infected mice (Fig. 2 and Table 2), but the intensities of PfMSP1 expression and DAPI staining (nuclear DNA) were lower in schizonts from iPfSPZ versus mPfSPZ (Fig. 2). The average 44.5% reduction in the numbers of late liver-stage schizonts in iPfSPZ-infected mice was not sufficient to explain the reduction in blood-stage infections. Thus, the transition to fully mature liver-stage schizonts and/or their rupture must be considerably diminished in iPfSPZ parasites.

Table 2 | Infectivity of iPfSPZ to human-liver chimeric FRGhuHep mice

	Experiment A		Experiment B	
	mPfSPZ	iPfSPZ	mPfSPZ	iPfSPZ
Number of PfSPZ injected	9.0×10^5	8.2×10^5	1.0×10^6	1.9×10^6
Number of parasites 10–20 µm expressing PfLSA1 per cm^2	174	109	296	219
Relative percentage conversion rate to PfLSA1-expressing parasites of iPfSPZ compared with mPfSPZ[a]		68%		38%
Number of parasites ≥30 µm expressing PfMSP1 per cm^2	9	5	11	8
Relative percentage conversion rate to PfMSP1-expressing parasites of iPfSPZ compared with mPfSPZ[a]		61%		29%

Livers removed 7 days after infection were frozen and 6 µm cryosections were examined for parasites by IFA using an anti-PfMSP1 monoclonal antibody and anti-PfLSA1 rabbit polyclonal antiserum. The total number of parasites with a diameter of 10–20 µm expressing PfLSA1 (early-to-late liver stage) and a diameter of ≥30 µm expressing PfMSP1 (late liver stage) were counted in 4 liver sections equivalent to about 1 cm^2 per mouse.

[a]The relative percentage conversion was calculated for each liver stage using the following formula:

$$\text{Relative percentage conversion rate} = \frac{(\text{no. iPfSPZ-derived liver parasites/no. iPfPSZ injected})}{(\text{no. liver stage parasites after mPfSPZ/no. mPfPSZ injected})} \times 100\%.$$

Entire *P. falciparum* life cycle without mosquitoes

Erythrocytic-stage parasites from the first FRGhuHep mouse infected with iPfSPZ produced *P. falciparum* gametocytes in vitro (Extended Data Fig. 2a,b), demonstrating the completion of the *P. falciparum* life cycle from gametocytes to gametocytes without mosquitoes. These iPfSPZ-derived *P. falciparum* gametocytes were fed in triplicate to *Anopheles stephensi* mosquitoes and, 7 days later, oocyst infection prevalence rates ranged from 24% to 82% with intensities of 0 to 28 oocysts per mosquito. Fifteen days after feeding, PfSPZ intensities in three containers were 2,144, 11,340 and 24,878 PfSPZ per mosquito, respectively (Extended Data Table 6).

Comparative gene and protein expression

We assessed mRNA expression using quantitative PCR with reverse transcription (RT–qPCR) analysis of the genes encoding four *P. falciparum* proteins expressed in PfSPZ in fresh, partially purified iPfSPZ from three independent samples from the supernatants of HFC cultures and in two independent samples of fresh, aseptic, purified mPfSPZ. The mRNA abundance of Pf*CSP*, Pf*SSP2* (sporozoite surface protein 2, also known as *TRAP*, thrombospondin-related anonymous protein), Pf*CelTOS* (cell-traversal protein for ookinetes and sporozoites) and Pf*AMA1* (apical membrane antigen-1) in iPfSPZ was 64%, 75%, 81% and 76% of that in mPfSPZ, respectively (Fig. 3a).

To further characterize iPfSPZ gene expression, we performed strand-specific RNA-sequencing (RNA-seq) analysis of partially purified iPfSPZ and highly purified salivary-gland-derived mPfSPZ. The reads for the libraries obtained from iPfSPZ aligned mostly with the *D. melanogaster* genome, due to the presence of S2 cells in the iPfSPZ cultures. The genes observed in the iPfSPZ transcriptome were identified previously as expressed in PfSPZ and their expression profile was very similar to the mPfSPZ. Our preliminary transcriptome analysis identified some differences in the expression levels of multiple genes in iPfSPZ versus mPfSPZ, but the overall expression profiles were similar (Pearson's $R = 0.896$; Spearman's $\rho = 0.914$) (Supplementary Table 4 and Extended Data Fig. 3a–c). Expression levels in iPfSPZ of the 50 genes most highly expressed in mPfSPZ are shown in Fig. 3b. Pf*CSP* was the most abundant transcript in mPfSPZ and iPfSPZ, and Pf*CelTOS* was the second and fourth most abundant transcript in mPfSPZ and iPfSPZ, respectively. Expression in iPfSPZ of Pf*CSP*, Pf*CelTOS*, Pf*SSP2* and Pf*AMA1* were 41%, 76%, 34% and 54%, respectively, of those in mPfSPZ. Of the 50 transcripts that were most highly expressed in mPfSPZ, 32 had greater expression in mPfSPZ and 18 had greater expression in iPfSPZ (Supplementary Table 5). Differential gene expression analysis identified 58 genes that were upregulated in iPfSPZ with a $\log_2$-transformed fold change of greater than 2. Among the genes more highly expressed in iPfSPZ were *PF3D7_1420500*, encoding a conserved protein of unknown function, *PF3D7_0202100*, encoding a liver-stage-associated protein 2 (LSAP2), and other transcripts associated with liver-stage development (Fig. 3b, Extended Data Fig. 3c and Supplementary Tables 4 and 5). iPfSPZ express a broadly similar transcriptome compared to mPfSPZ,

Table 1 | Infection of human-liver chimeric FRGhuHep mice with iPfSPZ or mPfSPZ by intravenous injection

Exp.	PfSPZ		Thin blood smear results for parasitaemia		
			Day 7 bleed of the infected mouse	In vitro blood stage parasite culture	
	Source	Number injected		Day 7	Day 19
1	mPfSPZ	9.0×10^5	Positive	Positive	Positive
	iPfSPZ	8.2×10^5	Negative	Negative	Positive
2	mPfSPZ	1.0×10^6	Positive	Positive	Positive
	iPfSPZ	1.9×10^6	Negative	Negative	Positive

On days 6 and 7 after injection, mice were infused with human blood and 7 h after the second blood infusion, blood from mice was transferred to *P. falciparum* culture and monitored for parasites by thin blood smear. Exp., experiment.

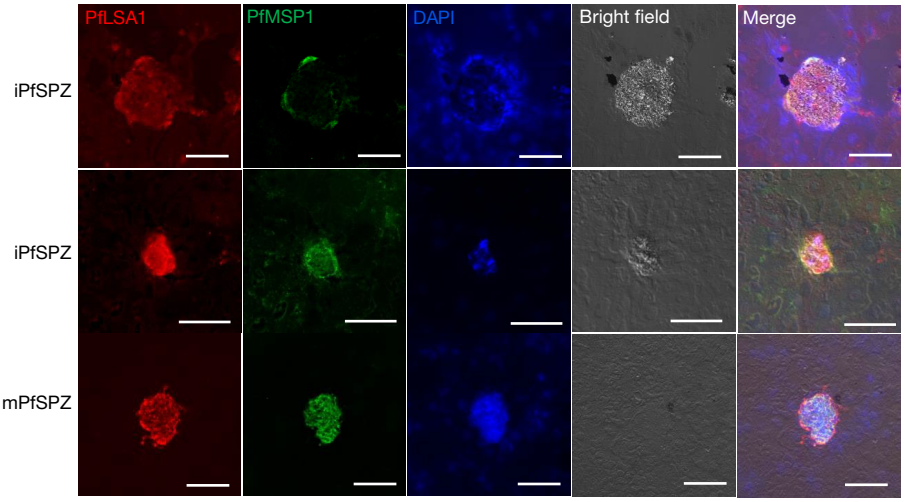

Fig. 2 | Infection by intravenous injection of a human-liver chimeric FRGhuHep mouse with iPfSPZ or mPfSPZ. Immediately after exsanguination in experiment 1 (Table 1), the liver was removed and cryopreserved at −80 °C and 6 µm cryosections were made. *P. falciparum* hepatocyte-stage parasites were detected by IFA using an anti-PfMSP1 monoclonal antibody and polyclonal anti-PfLSA1 rabbit antiserum by confocal microscopy. Scale bars, 50 µm.

but the data also show substantial differences in gene expression patterns. In particular, iPfSPZ show premature expression of some genes that are normally expressed only during the liver stages, possibly reflecting axenic transformation of the parasites in the cultures. Note that, due to the presence of S2 cells from the cultures, the majority of the RNA-seq reads were not parasite specific, so this analysis must be considered to be preliminary and not a full transcriptomic analysis given the low coverage obtained. Further optimization to purify iPfSPZ is required and is ongoing.

Immunoblot analysis was performed to compare the expression of PfCSP in iPfSPZ and mPfSPZ. The reduced expression of PfCSP in iPfSPZ compared with in mPfSPZ by RT–qPCR and RNA-seq was confirmed by immunoblot analysis of iPfSPZ extracted from a cell/oocyst pellet produced in an HFC (Extended Data Fig. 3d).

Immunogenicity of iPfSPZ versus mPfSPZ

Mice were immunized with aseptic, purified, cryopreserved mPfSPZ or partially purified iPfSPZ from the supernatants of HFC cultures. In humans, PfSPZ invade hepatocytes and induce T cell responses against liver-stage proteins expressed in infected hepatocytes, including some that are not expressed in PfSPZ. These T cell responses mediate protection conferred by attenuated SPZ in rodent and non-human primate malarias, but the protein targets of protective immunity have not been fully identified. IFNγ secreted by T cells and natural killer cells induces the production of nitric oxide by malaria-parasite-infected hepatocytes, which in turn is thought to kill the parasites[14,25–28].

The production of IFNγ by splenocytes was assessed using Fluorospot by culturing splenocytes from immunized mice with *P. falciparum*-infected red blood cells (PfRBCs)[15] as well as with mPfSPZ,

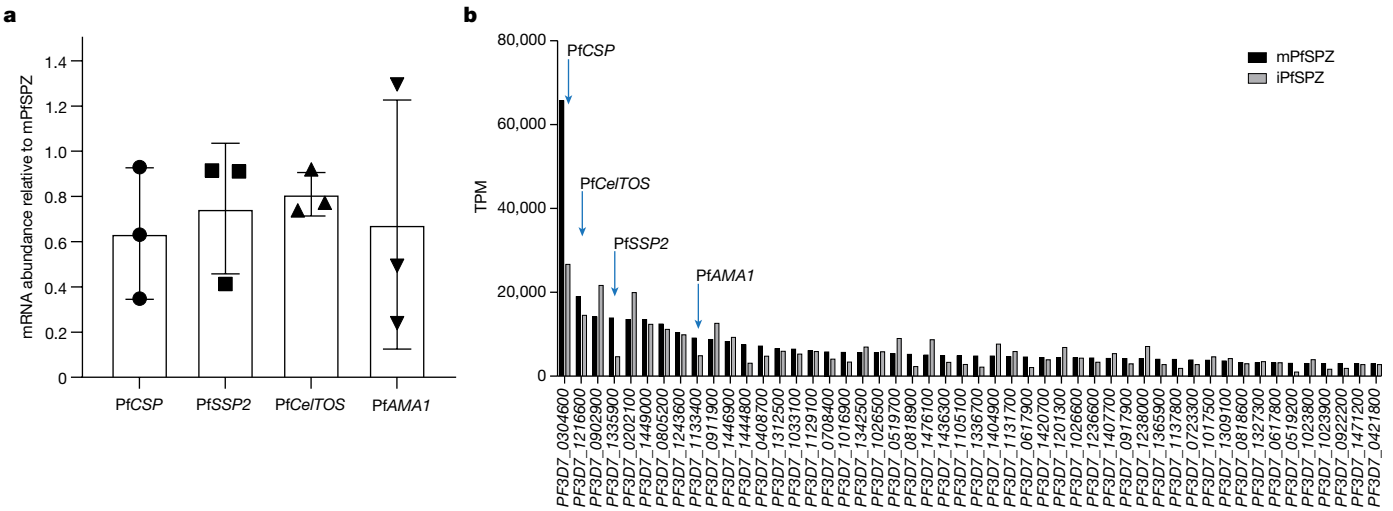

Fig. 3 | Gene expression in iPfSPZ versus mPfSPZ. a, Pf*CSP*, Pf*SSP2*, Pf*CelTOS* and Pf*AMA1* mRNA abundance in free iPfSPZ relative to mPfSPZ was analysed using RT–qPCR. mRNA abundance was determined using RT–qPCR normalized to *P. falciparum* 18S RNA. Data are the individual data points and mean ± s.d. from three independent biological replicates of partially purified iPfSPZ compared with the mean of two independent biological replicates of partially purified mPfSPZ. The mean values for the relative abundance of Pf*CSP*, Pf*SSP2*, Pf*CelTOS* and Pf*AMA1* were 0.63, 0.75, 0.81 and 0.68, respectively. **b**, RNA-seq analysis of the relative abundance of the 50 genes most highly expressed in mPfSPZ compared with those in iPfSPZ. Transcriptomes of duplicate biological samples of purified mPfSPZ and partially purified iPfSPZ were determined and the mean transcripts per million (TPM) (*y* axis) was plotted against the gene accession number. Pf*CSP*, Pf*CelTOS*, Pf*SSP2*, Pf*LSAP2 (PF3D7_0202100)* and Pf*AMA1* were expressed at significant levels in mPfSPZ and iPfSPZ transcriptomes. The results for all 50 genes are included in Supplementary Table 5.

recognizing that the mPfSPZ might induce better responses in mice immunized with mPfSPZ. The responses of freshly collected (and cryopreserved) splenocytes against PfRBCs were best at the highest antigen dose (2×10^5 PfRBCs) (Extended Data Fig. 4). For mice immunized with mPfSPZ and iPfSPZ, there was a median of 104 versus 78 spot-forming cells (SFCs) secreting IFNγ per million splenocytes ($P = 0.4$) respectively. At the highest stimulating dose (2.5×10^4 PfSPZ), responses to mPfSPZ stimulation were higher, but not significantly higher in mice immunized with mPfSPZ than iPfSPZ—18 versus 5 SFCs per million splenocytes, respectively—but lower than after PfRBC stimulation, (Extended Data Fig. 4).

Antibodies were assessed on a Pf3D7 proteome microarray[29]. Preliminary results indicated that mice that were immunized with mPfSPZ produced antibodies against more proteins and at higher antibody levels compared with mice that were immunized with iPfSPZ (data not shown).

Discussion

We produced PfSPZ in vitro that were infectious to human hepatocytes in culture and to mice with humanized livers in vivo. These results provide the foundation for optimizing the production and purification of iPfSPZ and the subsequent large-scale manufacturing of PfSPZ vaccines without mosquitoes, and will facilitate research into the biology of *P. falciparum* development from gametocytes to PfSPZ.

The transmission of *P. falciparum* begins with stage V gametocytes ingested by mosquitoes. The gametocytes transform into macrogametes and microgametes, and microgametes fertilize macrogametes to form zygotes. These then transform to ookinetes, which penetrate the midgut and form oocysts that are positioned in the extracellular space between the midgut epithelium and basal lamina. At 11–14 days after fertilization, oocysts rupture, releasing PfSPZ into the haemolymph, which then migrate to the salivary glands[30].

We first produced *P. falciparum* oocysts. The rate of conversion from stage V gametocytes to oocysts was substantially higher in vitro than in mosquitoes (Extended Data Table 2). Although the conversion from oocysts to PfSPZ was substantially lower in vitro, the overall conversion from gametocytes to PfSPZ was greater in vitro than in mosquitoes (Extended Data Table 1).

The highest conversion rates from stage V gametocytes to iPfSPZ occurred in cultures using Matrigel as a substrate matrix, followed by collagen-based matrices (Extended Data Fig. 1e and Extended Data Table 1). In the absence of Matrigel or collagen, 2.6-fold fewer iPfSPZ were produced (Extended Data Fig. 1e and Extended Data Table 1), meaning that the components of Matrigel or other matrices—such as laminin, collagen IV, heparan sulfate proteoglycans and entactin/nidogen—were not obligatory for development of infective iPfSPZ, but were potentially optimal. S2 cells, and presumably the nutrients or other molecules that they produced, improved production (Extended Data Fig. 1f,g and Extended Data Table 3). We produced iPfSPZ using culture materials that are compliant with GMPs, and there were no adverse outcomes when iPfSPZ produced without a matrix were injected into mice.

In culture, minimally purified, fresh and cryopreserved iPfSPZ invaded human hepatocytes and developed to liver-stage schizonts expressing a late liver-stage protein, PfMSP1, at rates as high as or higher than aseptic, purified, fresh and cryopreserved mPfSPZ (Fig. 1c,g and Extended Data Tables 4 and 5). However, in vivo in FRGhuHep mice with humanized livers, the numbers of late liver-stage parasites expressing PfMSP1 were about 2× lower for fresh iPfSPZ than for cryopreserved mPfSPZ. Notably, the conversion to infectious erythrocytic-stage parasites was substantially reduced with iPfSPZ (Table 1). Parasites derived from iPfSPZ appeared to be partially attenuated at the late liver stage. Recently, late-liver-stage-arresting, replication-competent PfSPZ have been generated by deleting genes that are critical for late liver-stage development[18]. iPfSPZ appear to show considerable intrinsic liver-stage attenuation, and this might constitute an added benefit in the production of liver-stage attenuated parasite vaccines.

We compared the differences in expression of mRNA using RT–qPCR and RNA-seq and PfCSP using immunoblotting between partially purified iPfSPZ and purified, cryopreserved mPfSPZ. Owing to the presence of S2 cells from the cultures, the majority of the RNA-seq reads were not parasite specific, so this analysis must be considered to be preliminary; we are working towards further analysis of more highly purified iPfSPZ preparations. Despite this limitation, the overall gene expression trends observed between iPfSPZ and mPfSPZ were maintained. In two biological replicates, there was a high correspondence between gene expression in mPfSPZ and iPfSPZ. Among the 50 genes that are most highly expressed in mPfSPZ, 18 (36%) had higher levels of expression in iPfSPZ (Fig. 3b and Supplementary Tables 4 and 5). Pf*CSP* expression was lower in iPfSPZ than in mPfSPZ—64% by RT–qPCR and 41% by RNA-seq (Fig. 3a)—and PfCSP expression was also lower at the protein level (Extended Data Fig. 3d). Our preliminary differential gene expression analysis showed that Pf*LSAP2*, encoding a protein implicated in pre-erythrocytic-stage protection[31], was more highly expressed in iPfSPZ than any gene except for Pf*CSP* in in mPfSPZ (Fig. 3b). In a preliminary single study, T cell responses against *P. falciparum*-infected erythrocytes were similar in mice immunized with iPfSPZ or mPfSPZ (Extended Data Fig. 4). Antibody responses are being further assessed, but were lower in magnitude and breadth compared with after mPfSPZ immunization. Further in-depth transcriptomic, proteomic and immunogenic characterization of iPfSPZ will be performed in the future on highly purified iPfSPZ optimized for development, quality, and magnitude and consistency of gene and protein expression.

P. falciparum sporogony requires nutrients, including lipids, which are necessary for parasite membrane biogenesis. These lipids are in part derived from the digestion of erythrocytes in the midgut and are acquired from the haemocoel through the mosquito lipid-transport machinery, including lipophorins. Lipids accumulate in the peripheral cytoplasm and vesicles of mature oocysts[32]. Depletion of lipophorin inhibits oocyst sporulation, causing abnormal cytoplasmic vacuolization and a significant reduction in the size of mature oocysts and the numbers of PfSPZ[33]. A probable reason for the lower transition from oocysts to iPfSPZ and the reduced gene expression in iPfSPZ compared to mPfSPZ was a lack of nutrients necessary for membrane biogenesis, particularly as the delivery of lipid-based nutrients in an aqueous medium is problematic. Furthermore, the oocyst capsule, which includes mosquito extracellular matrix proteins, especially laminin and collagen IV[34,35], and the oocyst outer membrane are critical for nutrient acquisition. We speculate that the absence of mosquito extracellular proteins in our cultures impaired the formation of the oocyst capsule, leading to suboptimal uptake of nutrients by oocysts in vitro, a lower conversion rate of oocysts to iPfSPZ, and reduced and inconsistent gene and protein expression.

We can produce large numbers of infectious iPfSPZ that fully develop in vitro and in vivo, but considerable work is still needed to optimize the quality, consistency and purity of the iPfSPZ, and their safety, immunogenicity and protective efficacy in humans before the potential of iPfSPZ is realized. PfNF54 and genetically attenuated PfNF54 (unpublished) iPfSPZ give similar results in our system, and we will assess other *P. falciparum* parasites in the future. The goal, which is now in sight, is the inexpensive, large-scale manufacture of the iPfSPZ needed for mass vaccination programs to eliminate *P. falciparum* malaria as a major cause of morbidity and mortality worldwide.

Online content

Any methods, additional references, Nature Portfolio reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41586-022-05466-7.

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Methods

Parasites, mosquitoes and feeder cells

P. falciparum strain NF54[36] was used for all of the experiments. Stage V *P. falciparum* gametocytes were produced in vitro using standard methods[37], and were fed to *A. stephensi* (SDA 500)[37,38] for in vivo production of PfSPZ in mosquitoes (mPfSPZ). The number of gametocytes ingested per mosquito was estimated on the basis of the percentage of erythrocytes infected with stage V gametocytes, the haematocrit of the blood meal preparation and an estimated 5 µl blood meal volume ingested by a mosquito. *D. melanogaster* Schneider S2 cells and *S. frugiperda*, Sf21 cells were purchased from Thermo Fisher Scientific.

Culture vessels, reagents and medium

Culture vessels. Lab-Tek 8-well chamber slides (Millipore), 12-well culture plates (Thermo Fisher Scientific), or 3.2 and 20 ml HFCs (FiberCell Systems) were used.

Matrix reagents. Matrigel was obtained from BD Biosciences, Culturex 3-D culture collagen I (rat tail) from Bio-Techne (3447-020-01) and GMP-grade bovine collagen from Collagen Solutions (CB001).

Exflagellation medium. Exflagellation medium was composed of heat-inactivated fetal bovine serum (91.7% (v/v); HI-FBS; (Gibco) containing 9.32 mM $NaHCO_3$, 1.9 mM xanthurenic acid and 2.9 mM glucose[39].

Ookinete medium. Ookinete medium was composed of RPMI 1640 medium supplemented with 10% HI-FBS, 14.6 mM trehalose, 4.7 mM sodium bicarbonate and 1% v/v penicillin–streptomycin (10,000 U ml^{-1} of penicillin and 10 mg ml^{-1} streptomycin; Gibco).

Oocyst medium

Matrigel-coated Lab-Tek slides and collagen-coated culture plates. Schneider's *Drosophila* medium (Thermo Fisher Scientific) supplemented with 10% v/v HI-FBS, 10% v/v spent medium from S2 cell culture (1×10^6 S2 cells per ml for 3 days), 14.6 mM trehalose (Alfa Aesar), 4.7 mM $NaHCO_3$ (Thermo Fisher Scientific) 100 U ml^{-1} penicillin, 0.1 mg ml^{-1} streptomycin, 10 mM HEPES (Gibco), 0.05 mM hypoxanthine (Acros Organics), 1.5% v/v lipoprotein cholesterol concentrate (ICN), and 44 nM para-aminobenzoic acid (PABA, Acros Organics).

Uncoated 12-well culture plates. Schneider's *Drosophila* medium supplemented with 15% v/v HI-FBS and 1% v/v penicillin–streptomycin 22–24 h after seeding of parasites.

Hollow fibre cartridges. Schneider's *Drosophila* medium (894 ml), 50 ml HI-FBS (5%), 30 ml CDM-HD (Chemically Defined Medium for High Density Cell Culture, Fiber Cell Systems), 10 ml penicillin–streptomycin (GIBCO), 10 ml of 1 M HEPES, 2 ml of 7.5% $NaHCO_3$ (GIBCO), 2 ml of 25 mM hypoxanthine and 0.2% (v/v) sugar cocktail (5 g trehalose anhydrous, 3 g glucuronic acid (Alfa Aesar), 1 g fucose (Thermo Fisher Scientific), 0.2 g arabinose (Thermo Fisher Scientific), 0.2 g xylose (Acros Organics), 0.2 g fructose (VWR chemical), 0.2 g mannose (VWR chemicals), 2 g inositol (Thermo Scientific), 0.2 g *N*-acetyl glucosamine (Spectrum Chemicals), 0.2 g glucosamine-HCL (Spectrum Chemicals), 25 ml water for injection grade, 1.5 ml 10 N NaOH (JT Baker), made up to 30 ml, aliquoted and stored at −20 °C).

Culture using Sf21 cells. Sf-900 II SFM medium (211.7 ml) (Thermo Fisher Scientific) was supplemented with 11.7 mM trehalose, 25 mM hypoxanthine, 10 mM HEPES, 40 nM PABA, 6.25 ml of 1% sterile peptone, 6.25 ml of 1% tryptone, 6.3 mM sodium bicarbonate, 2.5 ml of MEM amino acids solution (50×) (Thermo Fisher), 3.8 ml of EX-CYTE NZ growth enhancement medium supplement (Millipore Sigma) and 10 ml penicillin–streptomycin to a final volume of 250 ml then filter sterilized.

Production of gametes and zygotes

In vitro *P. falciparum* sporogony was initiated by resuspending stage V gametocytes in exflagellation medium. The tube containing the suspension was rocked gently at 26 °C for around 2 h allowing male and female gametes to emerge, and fertilization and formation of zygotes to occur. The culture was centrifuged at 1,800*g* for 3 min at ambient temperature and the contents were resuspended in ookinete medium.

Production of ookinetes

Eight-well chamber slides. Initial experiments used Lab-Tek 8-well chamber slides coated with Matrigel at 1 mg ml^{-1} in RPMI 1640 added at 4 °C, 400 µl per well and then incubated at 37 °C for 90 min. Excess liquid was removed from wells after polymerization of the Matrigel. The coated slides were washed once with 300 µl per well of ookinete medium, then seeded with 300 µl per well of S2 cells (or Sf21 cells) at 0.8×10^6 cells per ml in ookinete medium. A total of 2×10^3 to 2.5×10^4 stage V gametocytes that had been processed to produce gametes and zygotes (above) were then added to each well of the Lab-Tek chamber slides.

Twelve-well plates. Later experiments used 12-well culture plates. Rat tail collagen I or GMP-grade bovine collagen I was used as a matrix instead of Matrigel. For each, 0.3 mg ml^{-1} collagen was prepared in phosphate-buffered saline (PBS, pH 7.0, neutralized with 1 M NaOH) and 300 µl were added per well and the culture plates were incubated at 37 °C for 3 h. Excess liquid was removed from wells after polymerization of collagen. Other experiments did not use any matrices. Coated plates were washed once with 1 ml per well ookinete medium and then seeded with 500 µl per well of S2 cells at 0.8×10^6 cells per ml in ookinete medium. A total of 1×10^4 stage V gametocytes that had been processed to produce gametes and zygotes (above) were added to each well of the 12-well plates.

Production of oocysts and sporozoites

Chamber slides and plates. Ookinete medium was replaced with oocyst medium 22–24 h after addition of parasites. Cultures in 8-well chamber slides (300 µl per well medium) were maintained at 26 °C with normal air. Cultures in 12-well plates (3.0 ml per well medium) were maintained at 26 °C inside a modular incubator chamber flushed with a gas mixture of 15% O_2, 5% CO_2 and 85% N_2 at a flow rate of 20 l min^{-1} for 4 min after each medium change and incubated. For both, medium was changed every 48–72 h and the cultures were maintained for 14–18 days.

Hollow fibre cartridge. The above methodology was adapted to 3.2 ml and 20 ml polysulfone HFCs. The 3.2 ml cartridges were primed with PBS or RPMI for 24 h (ref. [40]) using a Duet pump[41] (speed rate of 12) to circulate medium through the cartridge fibres from a reservoir. The cartridges were then equilibrated using ookinete medium (250 ml reservoir volume) for at least 6 h, before seeding with 5 ml of ookinete medium containing 1×10^7 S2 cells and zygotes and gametes transformed from 6×10^6 stage V gametocytes (for 3.2 ml cartridge) or 5×10^7 S2 cells and zygotes and gametes transformed from 2.5×10^7 stage V gametocytes (for 20 ml cartridge) added to the extracellular space (ECS) of the cartridge. Ookinete medium in the reservoir was replaced with 250 ml of fresh ookinete medium and circulated as described above then, 22–24 h later, replaced with 250 ml (500 ml for 20 ml cartridge) of oocyst medium. The culture was maintained until collection (24–30 days after seeding) with replacement of supplemented oocyst medium every 3–4 days.

Assessment of ookinete motility

Evidence of ookinete motility was assessed in parasites cultured on Matrigel-coated 8-well chamber slides 24 h or 48 h after seeding gamete and zygote preparations. Slides were washed in PBS, fixed using

4% paraformaldehyde for 1 h, washed three times in PBS, blocked and permeabilized using 2% bovine serum albumin in PBS containing 0.1% Triton X-100 for 1 h. They were then incubated in 3.68 μg ml^{-1} of anti-Pfs25 monoclonal antibodies[22] (MRA-315, MR4) in blocking buffer, incubated for 2 h at 37 °C and reactivity was detected by incubation with Alexa Fluor 488 anti-mouse IgG (Thermo Fisher Scientific). Trails of Pfs25 detected on the slide surface demonstrated the path of movement of an ookinete.

Detection of 3- and 8-day oocysts

Oocysts were detected in 3-day or 8-day in vitro cultures in 8-well chamber slides by washing in PBS, then fixing, blocking and permeabilizing as described above. The samples were incubated with MRA-315 to detect Pfs25 in 3-day oocysts and with monoclonal antibody A210 to detect PfCSP[42] in 8-day oocysts. Reactivity was demonstrated using Alexa Fluor 488 anti-mouse IgG. Inconsistent background fluorescence at times made differentiation between 8-day oocysts and S2 cells difficult, causing potential overestimates of the numbers of 8-day oocysts.

We conducted experiments with Sf21 cells and Matrigel (n = 7), S2 cells and Matrigel (n = 23), collagen 1 (n = 24) or GMP collagen 1 (n = 11) and S2 cells alone (n = 37). Data were compared using Kruskal–Wallis tests of median values (P < 0.0001). Post hoc pairwise comparisons were performed using Dunn's multiple-comparisons test.

Partial purification of iPfSPZ

Culture material from 8-well chamber slides (~0.3 ml per well) were transferred to 1.5 ml tubes and triturated using three 1 ml syringes (0.5 ml per syringe) with a 25-gauge needle to release iPfSPZ by passing the culture material through the needle 15 times. Twelve-well plate cultures were transferred to a 50 ml Falcon tube. Each tube was centrifuged for 10 min at 1,000g (Eppendorf Centrifuge 5810 R with A-4-62 Rotor). The pellet from each tube was resuspended in 5 ml DTP (dissection, trituration and purification) medium and transferred into GentleMACs C tubes (Miltenyl Biotec) and dissociated using cycle B of the GentleMACs Dissociator (Miltenyi Biotec) to release iPfSPZ. To remove debris, dissociated extracts were centrifuged at 1,000g for 10 min. The supernatant was saved, and the pellet was washed with 5 ml DTP medium, the tube was centrifuged at 1,000g for 10 min and the pellet was discarded. Pooled supernatants were transferred to Oak Ridge tubes (Thermo Fisher Scientific) and centrifuged at 27,620g for 20 min (Sorvall RC+ Centrifuge, HB-6 Rotor). Pellets containing iPfSPZ were pooled and resuspended in 1–3 ml DTP medium.

iPfSPZ from HFC cultures were collected by removing the contents from the ECS containing free iPfSPZ, oocysts and S2 cells. Typically, 30–50 ml (3.2 ml cartridge) and 120–150 ml (20 ml cartridge) of culture were removed by repeatedly collecting the contents of the ECS according to the manufacturer's instructions. Collected samples were diluted by adding 0.5 volumes DTP, the samples were mixed by inversion and then centrifuged (Eppendorf 5810 R) at 800g for 10 min. Free iPfSPZ were separated in the supernatant from the majority of S2 cells and oocysts (pellet). The pellet was suspended in 15–30 ml DTP medium and the sample was centrifuged at 800g to remove iPfSPZ trapped in the pellet. The supernatants were pooled and the number of free iPfSPZ was determined by counting using a Cellometer counting chamber (Nexcelom). This preparation contained substantial amounts of S2 cell debris and was used to provide material for RT–qPCR, RNA-seq, cryopreservation, in vitro potency and immunization studies. Alternatively, the pellet containing ~10,000 oocysts and S2 cells combined in 5 ml DTP medium was dissociated using a GentleMACs Dissociator (see above).

Counting

iPfSPZ suspended in DTP were diluted as needed, and 20 μl was loaded into a Cellometer counting chamber. The number of iPfSPZ in each of the four quadrants observed using phase-contrast microscopy were used to calculate the concentration (iPfSPZ per ml = mean number of iPfSPZ per quadrant × dilution × 10^4) and the total number of iPfSPZ. iPfSPZ were counted only in unpurified or partially purified samples and were at times difficult to distinguish from extraneous culture materials. Therefore, counts of iPfSPZ may be overestimates.

Cryopreservation

iPfSPZ were centrifuged at 27,620g for 20 min (Sorvall RC+ Centrifuge, HB-6 Rotor), resuspended and then adjusted to a concentration of 1×10^8 iPfSPZ per ml. An equal volume of 2× cryoprotectant additive was added and mixed using a pipettor, the iPfSPZ suspension was dispensed into cryovials (Thermo Matrix) at 20 μl per vial (10^6 iPfSPZ per vial), rate-frozen (Planer Products Kryo16) and stored in liquid nitrogen vapour phase below −150 °C. For thawing, the lower half of the cryovial was immersed for 30 s in 37 °C water in a circulating water bath, and the appropriate medium or diluent added (see below). Further dilutions were made as appropriate for the particular assays.

Six-day hepatocyte potency assay

The potency of fresh and cryopreserved iPfSPZ and mPfSPZ was compared in the 6-day hepatocyte potency assay[43]. HC-04 cells[44] were seeded into 8-well chamber slides (4×10^4 cells/well) then, 24 h later, 5×10^4 iPfSPZ or mPfSPZ were added to triplicate wells. After 3 h, free PfSPZ were removed by washing, then the culture was incubated at 37 °C for 6 days with medium (DMEM/F-12 medium with 10% FBS and 2% penicillin–streptomycin) changed every 24 h. Six-day parasites were detected by immunofluorescence using a mouse monoclonal antibody against PfMSP1[45] (74.6 μg ml^{-1}) and Alexa Fluor 488 anti-mouse IgG (Thermo Fisher Scientific) and enumerated as described[14,43]. PHHs were also used to determine the potency of pooled dissociated iPfSPZ and purified cryopreserved mPfSPZ. Cryopreserved PHHs from male donors were obtained from BioIVT, thawed and approximately 1.5×10^5 cells per well were seeded into six-well chamber slides in steroid-free, cryoplateable medium (BioIVT). Dissociated iPfSPZ or purified, cryopreserved mPfSPZ suspended in DTP medium were seeded at 5×10^4 per well. Slides were gently agitated to disperse the PfSPZ and incubated at 37 °C. Then, 3 h later, PHH cryoplateable medium was changed and the culture was incubated for another 6 days with the medium changed every day. Developing 6-day parasites were detected by immunofluorescence using mouse monoclonal antibodies against PfMSP1 (as above), the *P. falciparum* 70-kDa heat shock protein (PfHSP70)[46] (69.2 μg ml^{-1}) or PfEXP1[47] (81.6 μg ml^{-1}) followed by Alexa Fluor 488 anti-mouse IgG, and then enumerated. Furthermore, we assessed the expression of PfLSA1 (1:50 dilution) using a rabbit polyclonal antiserum[48]. The mean numbers of 6-day parasites from triplicate wells were calculated. Wells containing HC-04 or PHH cells not seeded with PfSPZ were used as negative controls.

Infection of FRGhuHep mice

Female 6–8-week-old FRG-knockout mice on the NOD background with human chimeric livers (FRGhuHep mice) were purchased from Yecuris Corp (Tualatin) and housed at Bioqual, Maryland, at 65–75 F (18–24 °C) and 45–50% humidity and under a 12 h–12 h dark–light cycle, and all of the protocols were approved by the Bioqual Institutional Animal Care and Use Committee (IACUC). All of the mice had human liver repopulation levels above 70%. Established protocols for injecting the mice with PfSPZ were followed[49]. Partially purified iPfSPZ produced in 12-well plates without a matrix or aseptic, purified, cryopreserved mPfSPZ as positive controls were injected intravenously into randomly allocated, single mice in two experiments (Table 1). On days 6 and 7 after injection, the mice were infused intravenously with 400 μl packed human O^+ erythrocytes and injected intraperitoneally with 200 μl penicillin–streptomycin (100 U of penicillin and 100 μg of streptomycin per ml). Then, 7 h after the second infusion, blood was collected by cardiac puncture, the mice were euthanized, and livers were collected and immediately frozen at −80 °C. Technical staff were blinded to whether mice received mPfSPZ or iPfSPZ.

Entire *P. falciparum* life cycle without mosquitoes

A sample from the cardiac puncture of each infected FRGhuHep mouse was assessed by reading a Giemsa-stained thin blood smear at ×1,000 magnification. The rest of the blood was added to 10 ml complete medium (RPMI 1640 with 25 mM HEPES and 2 mM L-glutamine (Mediatech), 10% human O^+ serum (Interstate Blood Bank), 33.3 μM hypoxanthine (VWR) and 25 μg ml^{-1} gentamicin (Gibco) and pelleted by centrifugation at 200*g*. The supernatant and the buffy coat (containing white blood cells) were removed. The erythrocytes were then washed three times with 10 ml complete medium, with pelleting and centrifugation as described above. After the third wash, an equal volume of packed human O^+ erythrocytes (hrbc) (approximately 400 μl) was added, and the erythrocyte pellet was resuspended in complete medium to 1% haematocrit. Cultures were seeded into wells of 6-well plates (5.0 ml per well at 1% haematocrit in complete medium) and maintained in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2. The culture medium was exchanged daily and 50 μl of fresh packed hrbc was added every 5 days to each well. Thin blood smears of cultures were made daily, Giemsa-stained and read by microscopy. The culture was expanded once parasitaemia reached 1% by the addition of hrbc. Then, 9, 11 and 13 days after the first positive thin blood smear (Table 1 (experiment 1)) the cultures were induced to produce gametocytes by adding hrbc to achieve a 5% haematocrit and 0.61%, 0.74% and 0.5% parasitaemia (mixed stages), respectively, and were maintained for 17 days with daily medium changes without the addition of hrbc. For blood from one of the mice (Table 1, experiment 1) at day 18 after induction, an artificial blood meal was prepared by combining cultured stage V gametocytes with uninfected hrbc to a final gametocyte concentration of 0.14–0.16% and O^+ human serum to achieve a haematocrit of 50%. Adult female *A. stephensi* mosquitoes 3–7 days after emergence were fed on the blood meal through an artificial membrane for 30 min. Blood fed mosquitoes were maintained at 26 °C, 75% humidity, and were provided with sugar cubes and water ad libitum. Infection prevalence and intensity were assessed on day 7 after feeding by examining dissected mercurochrome-stained midguts by light microscopy for the presence of oocysts, and on day 15 or 16 by dissection of salivary glands and scoring sporozoite intensities.

Liver infections in FRGhuHep mice

Sections (6 μm) of frozen livers were mounted on microscope slides and *P. falciparum* liver stage parasites assessed by IFA using the anti-PfMSP1 monoclonal antibody as described for hepatocyte cultures above[14,43] and polyclonal anti-PfLSA1 rabbit antibodies as described previously[14]. The total number of parasites of 10–20 μm in diameter expressing PfLSA1 (early-to-late liver stage) and ≥30 μm diameter expressing PfMSP1 (late liver stage) were counted in 4 liver sections equivalent to ~1 cm^2 per mouse.

Gene expression studies

RT–qPCR. Approximately 3×10^6 freshly prepared, partially purified iPfSPZ from the supernatants of HFC cultures or freshly dissected, aseptic, purified mPfSPZ were washed using PBS and the pellets were stored at −80 °C. Total RNA was extracted using the QIAzol lysis reagent (Qiagen) and genomic DNA was removed using DNase I (New England Biolabs), both according to manufacturer's instructions. cDNAs were synthesized from 1 μg total RNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) using random primers. qPCR reactions were performed on cDNA using the SensiFAST Real-Time PCR Kit (Bioline). The *P. falciparum* 18S rRNA gene was used for normalization. The following forward and reverse primers were used.

Pf*CSP*, TCAACTGAATGGTCCCCATGT and GAGCCAGGCTTTATTCTAACTTGAAT[50]; Pf*SSP2* (also known as *TRAP*), CCAGAAGAAGGAAAGGGTGAA and TTTGGTGGATTTGGTGGATTT; Pf*CelTOS*, CGACCCAGCAACATATGGTATA and TTCTGAGACGTCTGGTGAATTG; Pf*AMA1*), CGCATATCCAATAGACCACGA and CCGTCCATGGATTACCCATATAA; 18S rRNA, AGAATTGACGGAAGGGCAC and ACTAGTGAGTTTTCCCGTGTTG[51].

RNA-seq library preparation and data analysis. Total RNA from iPfSPZ from the supernatants of HFC cultures or fresh, aseptic, purified mPfSPZ was extracted using the miRNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions, including on-column DNase digestion. Total RNA was poly(A)-enriched using the Dynabeads mRNA Purification Kit (Life Technologies) and a strand-specific RNA-seq library prepared using the TruSeq Stranded mRNA Library Prep Kit (Illumina)[52]. The libraries were multiplexed and processed for 75 bp paired-end sequencing on the Illumina NextSeq 500 system. The resulting data were demultiplexed using bcl2fastq2 (v.2.20, Illumina) to obtain fastq files for downstream analysis. A minimum of two biological replicates was analysed for both iPfSPZ and mPfSPZ. Sequencing reads were mapped to a combined index of three genomes (*P. falciparum*[53] (PlasmoDB, v.32, https://plasmodb.org/plasmo/app/downloads/release-32/PfalciparumD7/); *D. melanogaster*[54] (BDGP6.32, https://nov2020.archive.ensembl.org/Drosophila_melanogaster/Info/Index); *A. stephensi* (UCIrvine; v.1.0, https://www.ncbi.nlm.nih.gov/genome/2653?genome_assembly_id=985930)) with the Bowtie2 Alignment tool[55] (v.2.3.4.2) using the default parameters with the effort options set to '--very-sensitive'. Reads not aligning to the combined genomes index were then mapped to a combined index of all expected exon splice boundaries from the three genomes, to capture reads spanning exons. The BAM files were then combined into read depth wiggle tracks that record both uniquely mapped and multiply mapped reads to each of the forward and reverse strands of the genomes at single-nucleotide resolution. Multiple mapped reads were pro-rated over all highest quality aligned locations.

Gene sequences were filtered to remove non-strand-specific read artifacts. Strand-specific pileup images of genes showing an unexpected proportion of reads aligning to the non-coding strand were manually inspected. From those, 69 genes were flagged as having anomalous alignment artifacts not consistent with expected RNA-seq expression profiles and were excluded from downstream analyses.

Gene expression quantification was then measured by summing total reads landing inside annotated gene exon boundaries, expressed as reads per kilobase of transcript, per million mapped reads (RPKM)[56] transcripts per million (TPM)[57] and raw read counts. Gene expression and differential gene expression analysis were performed using the R package DuffyNGS (v.1.9.1, https://github.com/robertdouglasmorrison/DuffyNGS). To minimize biases from the choice of algorithm for calling differentially expressed (DE) genes, a panel of five DE tools was used. They include: (1) Round Robin (in-house); (2) Rank Product[58]; (3) Significance Analysis of Microarrays (SAM)[59] (R package siggenes, v.1.58.0); (4) EdgeR[60] (v.3.26.8); (5) DESeq2[61] (v.1.24.0). Each DE tool was called with the appropriate default parameters, and operated on the same set of transcription results using: TPM expression units for RoundRobin, RankProduct and SAM; and raw read count units for DEseq2 and EdgeR. All 5 DE results were then synthesized into one result by combining fold changes, *P* values and rank positions from all five DE tools. Specifically, the rank position of a gene in all five results is averaged, using a generalized mean to the 1/2 power (square root mean), to yield the final net rank position of the gene. The explicit measurements of fold change and significance (*P* value) are similarly combined by appropriate averaging (arithmetic and geometric mean, respectively).

Immunoblot analysis of iPfSPZ and mPfSPZ

Cryopreserved, aseptic, purified mPfSPZ and partially purified iPfSPZ dissociated from the pellets of HFC cultures were run on 4–20% Tris glycine gels (Thermo Fisher Scientific) using Novex Sharp Pre-Stained Protein Standards (Thermo Fisher Scientific), and run in tris glycine running buffer (Thermo Fisher Scientific). The samples were transferred for 7 min from the gel onto a nitrocellulose membrane (Thermo Fisher Scientific). The membrane was blocked with 5% dried skim milk

(Lab Scientific bioKEMIX) in Tris-buffered saline with 0.2% Tween-20 and probed with anti-PfCSP monoclonal antibody 2A10 at 1:500 dilution (6.86 μg ml^{-1}). The secondary antibody was anti-mouse IgG conjugated to alkaline phosphatase, diluted 1:5,000 (Thermo Fisher Scientific).

Preparation of PfSPZ for immunization

iPfSPZ were produced in HFCs. iPfSPZ from the supernatant after 800*g* centrifugation were enriched by passing through a 0.3 μm disposable capsule filter (Millipore). Aliquots of filtrate containing 1×10^6 iPfSPZ were added to 1.5 ml tubes and centrifuged at 15,000 rpm for 6 min. Pellets containing iPfSPZ were washed twice using M199, 10% normal mouse serum (NMS). The pellet containing 1×10^6 iPfSPZ was suspended in 200 μl M199, 10% NMS for injection. Aseptic, purified, cryopreserved mPfSPZ were thawed and 1×10^6 mPfSPZ were washed in M199 medium then in M199, 10% NMS serum and the mPfSPZ pellet was suspended in 200 μl M199 with 10% NMS for injection.

Immunization of mice

BALB/c mice (aged 6 to 8 weeks) from Envigo were housed under a 12 h–12 h light–dark cycle, between 65 and 75 F (~18–23 °C) under 45–50% humidity at Bioqual, and all of the protocols were approved by the Bioqual IACUC. Group sizes of 6 mice were chosen on the basis of our experience with these types of experiments, and mice were allocated randomly to treatment groups. Mice were immunized intravenously with iPfSPZ or mPfSPZ. Four doses of 1×10^6 PfSPZ in 200 μl per mouse were administered on days 1, 15, 29 and 48. Mice were bled (submandibular) for serum collection before immunization and ~2 weeks after the third immunization dose. Bleed volumes and schedules were performed according to best practices[62]. Terminal blood draw and organ collection were performed on day 57 after infection.

T cell studies

Antigen-specific immune splenocytes secreting IFNγ were evaluated using precoated FluoroSpot plates and kits (Mabtech) according to the manufacturer's instructions. Freshly collected and cryopreserved splenocytes from mice immunized with iPfSPZ or mPfSPZ or naive mice were suspended at 4×10^6 cells per ml in 100 ml complete medium (RPMI-1640 supplemented with 1% penicillin–streptomycin, 1% L-glutamine (Gibco) and 10% FBS (Sigma-Aldrich), and incubated in FluoroSpot plates with stimulants: PfRBC stimulation with 2×10^5, 1×10^5 and 5×10^4 PfRBCs per well was assessed. PfSPZ stimulation with 2.5×10^4 PfSPZ per well was assessed. As controls 2×10^5, 1×10^5 and 5×10^4 uninfected RBCs per well were used to stimulate splenocytes from immunized mice. Unstimulated splenocyte wells received diluent only as negative controls. Cultures were incubated for 40–42 h at 37 °C in 5% CO_2. Each splenocyte sample was assayed in duplicate and the number of single-staining IFNγ secreting cells were reported as SFCs and enumerated using an automated FluoroSpot reader (AID iSpot). Assay staff were blinded to treatment group.

Reproducibility

For Fig. 1a, PfCSP-expressing iPfSPZ were reproduced in cultures using 8-well chamber slides with Matrigel, 12-well culture plates with collagen matrices and without collagen, and using HFCs. The result from a 12-well culture plate culture with collagen-matrix-produced PfCSP-expressing iPfSPZ is shown in Fig. 1a.

For Fig. 2b, for the 6-day liver-stage schizonts in HC-04 cells after infection with iPfSPZ, schizonts in HC-04 cells expressing PfMSP1 were reproduced using iPfSPZ produced by 8-well chamber slides with Matrigel, 12-well culture plates with and without collagen matrices and using HFCs. Confocal micrographs were taken in an HC-04 cell assay for iPfSPZ produced using 12-well culture plates (twice) and 8-well chamber slides with Matrigel (once).

For Fig. 1d–f, confocal micrographs were taken of 6-day liver stage parasites in PHHs after infection with iPfSPZ and detected by IFA using anti-PfLSA1 polyclonal serum, anti-PfEXP1 monoclonal antibodies and anti-PfMSP1 monoclonal antibodies. This assay was performed twice using 12-well-culture-produced iPfSPZ and confocal micrographs were taken only once.

For Fig. 2, micrographs were taken only once.

For Fig. 3b, gene expression in iPfSPZ and mPfSPZ was determined by RNA-seq using two independently produced biological samples of iPfSPZ and mPfSPZ.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The NCBI BioProject accession number for the RNA-seq fastq files generated in this study is PRJNA753927. Details on how to use the files are provided in Supplementary Table 4. Genomes were obtained from PlasmoDB v.32 (https://plasmodb.org/plasmo/app/downloads/release-32/Pfalciparum3D7/); BDGP6.32, https://nov2020.archive.ensembl.org/Drosophila_melanogaster/Info/Index); and UC Irvine for *A. stephensi* (v.1.0, https://www.ncbi.nlm.nih.gov/genome/2653?genome_assembly_id=985930).

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Author contributions S.L.H. led the entire project. A.G.E., S.C., P.F.B., B.K.L.S. and S.L.H. designed the studies. A.G.E., M.M., H.H., I.M. and A.A.Y. performed in vitro culture experiments and data collection. P.D.L.V., B.U.H. and A.R. performed in vitro infectivity assays using HC-04 cells and data collection. P.D.L.V., H.H., H.K. and A.G.E. performed in vitro infectivity assays using PHHs and data collection. S.C., N.K., T.L., C.T. and A.G.E. performed FRG mouse infection studies and data collection. T.L., A.P., Y.A., C.T. and B.K.L.S. produced stage V gametocytes. T.L., A.G.E., I.M., H.H., C.T., B.U.H. and S.C. performed *P. falciparum* life cycle without mosquitoes and data collection. G.Z., R.D.M. and S.H.I.K. performed transcriptome analysis and data collection. E.I. performed RT–qPCR. M.B. and M.S. performed T cell studies and data collection. S.C. and N.K. immunized mice. J.J.C. performed proteome assays. A.S.I.A., M.L. and T.W. performed immunoblot analysis. A.G.E., P.F.B., B.K.L.S. and S.L.H. wrote the manuscript. B.K.L.S. and S.L.H. supervised the project. All of the authors discussed the results and commented on the manuscript.

Competing interests A.G.E., S.C., N.K, A.P., Y.A., A.A.Y., E.I., A.S.I.A., T.W., M.L., P.F.B., B.K.L.S. and S.L.H. are employees of Sanaria. B.K.L.S. and S.L.H. own stock in Sanaria. US patents on in vitro PfSPZ, 9878026B2 (2018) and 10441646B2 (2019) have been issued (inventors A.G.E. and S.L.H.). T.L., M.M., H.H., C.T., I.M., A.R. and P.D.V. were employees of Sanaria at the time the study was conducted.

Additional information

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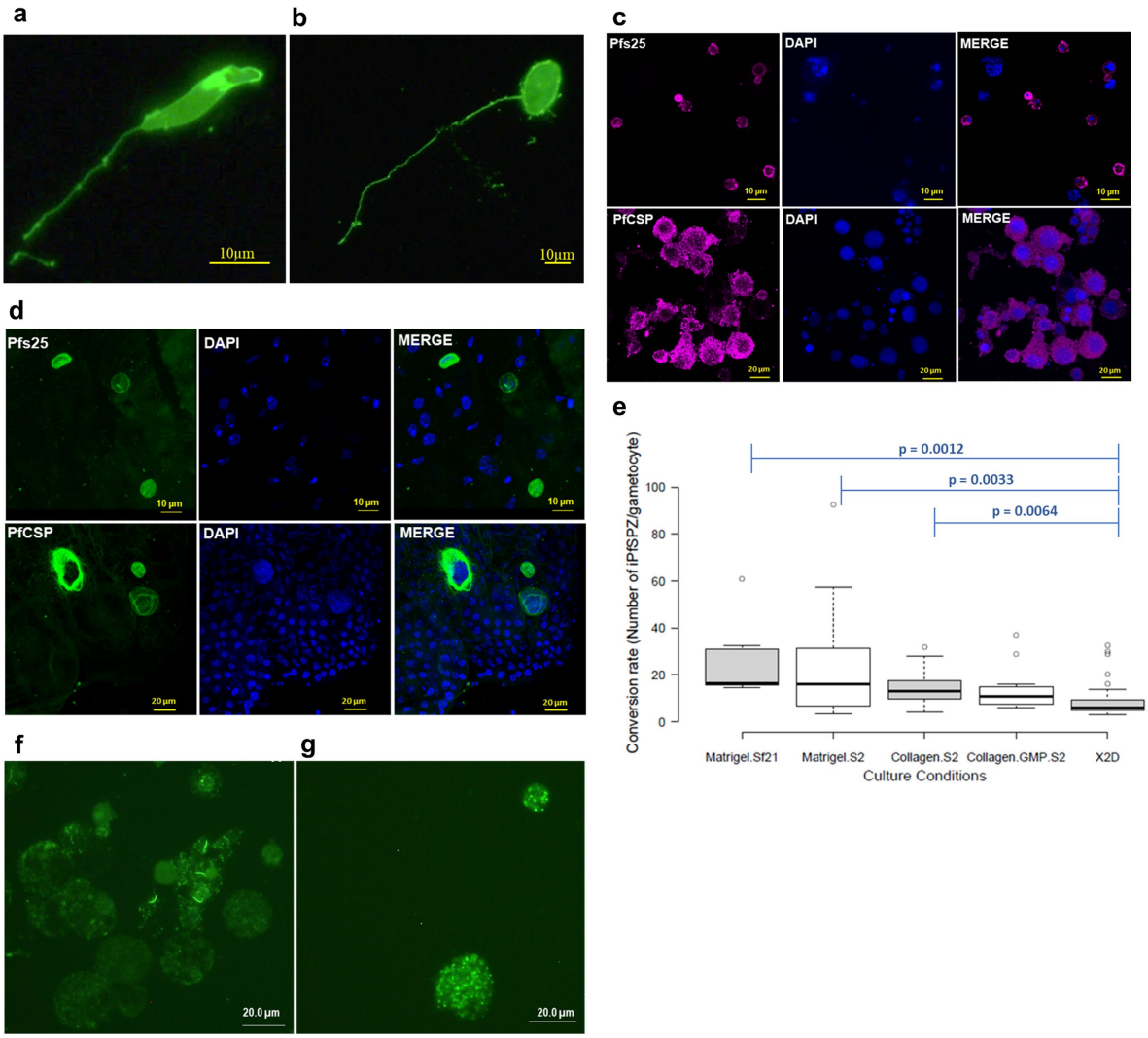
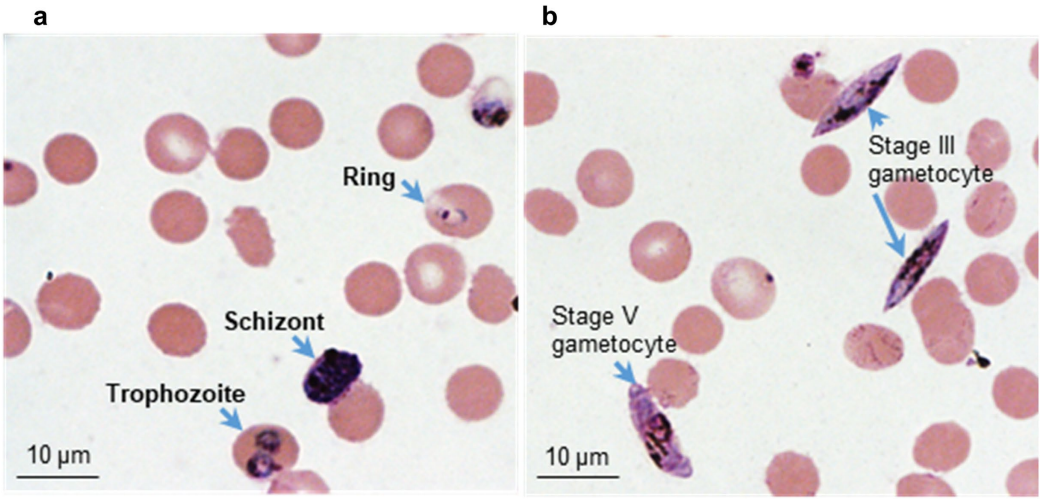
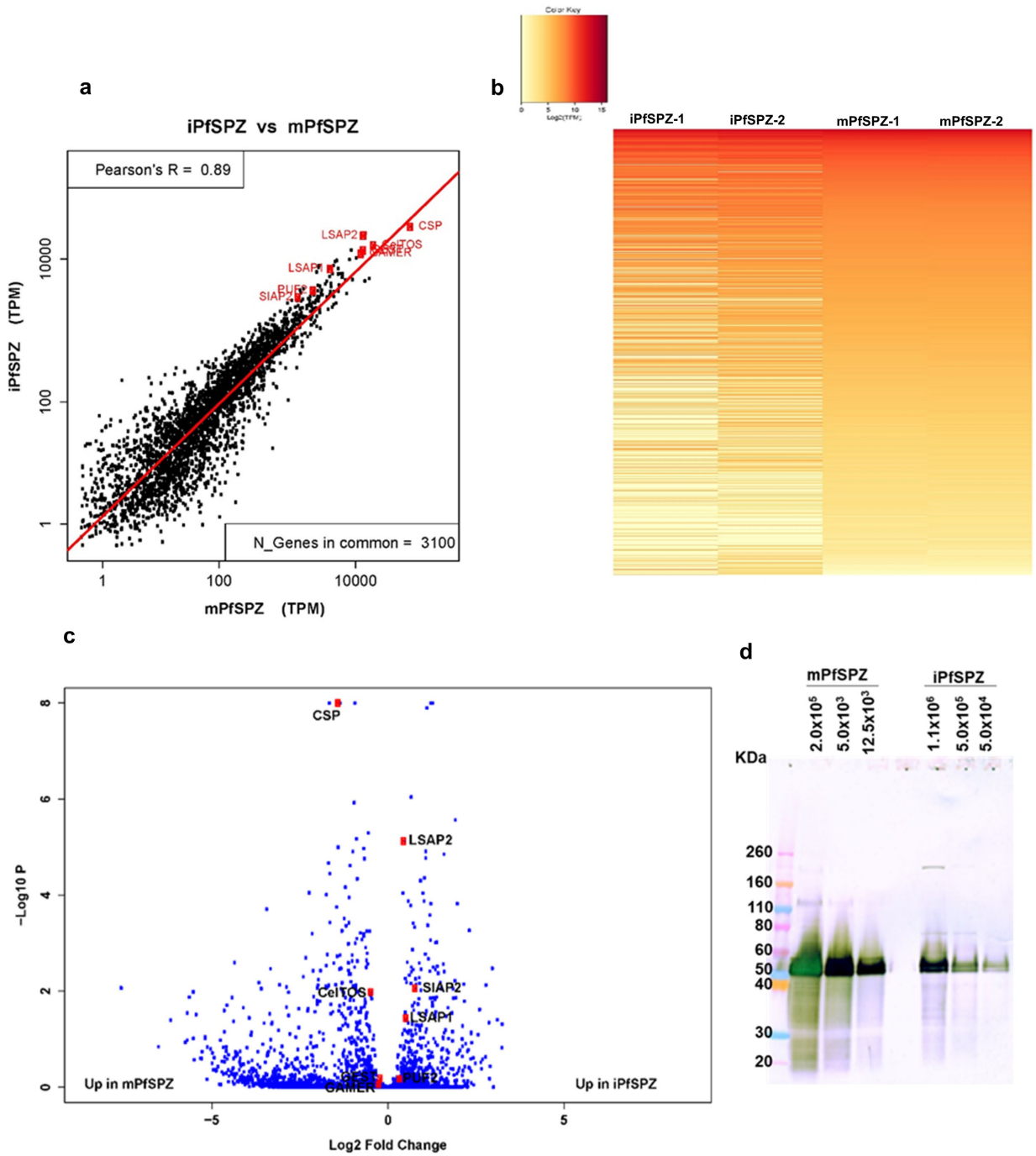


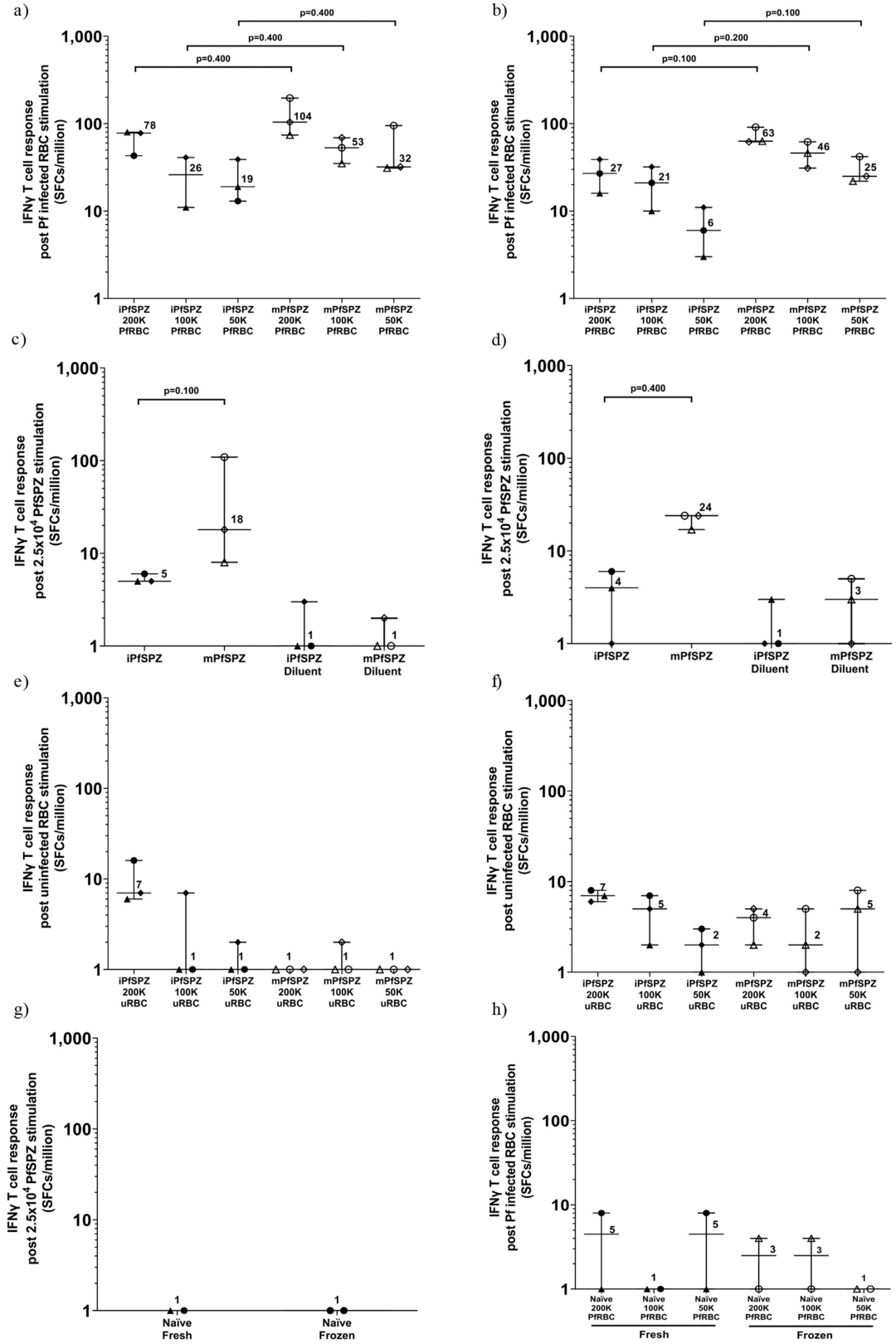
Extended Data Fig. 1 | Ookinetes and oocysts *in vitro*. a, b. *In vitro* transformed Pf zygotes and S2 feeder cells were added to Matrigel matrix coated wells of 8-well chamber slides and 24 h (**a**) or 36 (**b**) later an anti-Pfs25 mAb was added to the wells and the parasites were assessed by immunofluorescence assay. **a**. At 24 h, Pfs25 staining of an ookinete and the trail it had shed moving across the well are shown. **b**. At 36 h, Pfs25 staining of an early oocyst and the trail it had shed indicating the likely path the ookinete took before transforming to an oocyst are shown. Scale bars, 10 µm. Ookinetes expressing Pfs25 in 8-well chamber slide Matrigel cultures were identified in 3 different experiments. **c**. At 3 days (upper panels) an anti-Pfs25 mAb or at 8 days (lower panels) an anti-PfCSP mAb was added to the cultures and oocysts were assessed by immunofluorescence using confocal microscopy. Punctate localization in 8-day oocysts indicates budding PfSPZ. DAPI signals not localized to oocysts indicate feeder cell nuclei. *In vitro* produced 3-day oocysts expressing Pf25 and 8-day oocysts expressing PfCSP were detected in numerous experiments in 8-well chamber slides with Matrigel. **d**. *A. stephensi* mosquitoes were fed a blood meal containing stage V Pf gametocytes. At 3 days (upper panels) and 8 days (lower panels) the midguts were dissected and stained with anti-Pfs25 mAb (upper panels) and anti-PfCSP mAb (lower panels) and assessed by immunofluorescence using confocal microscopy. **e**. Comparison of conversion rates of gametocytes to iPfSPZ in different culture conditions. Center lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by dots. There were 7, 23, 24, 11, and 37 different independent experiments respectively for 8-well chamber slides with Sf21 cells, 8-well chamber slides with Matrigel, 12-well plates with S2 cells and collagen I, 12-well plates with S2 cells and GMP collagen, and 12-well plates with S2 cells and no matrix. All data were compared using Kruskal-Wallis test of median values (<0.0001), and post-hoc pairwise comparisons were made using Dunn's multiple comparisons test. Significant differences and their respective p values are shown on the figure. **f-g**. *In vitro* transformed Pf zygotes (the product of $2x10^3$ stage V gametocytes) with (**f**) and without (**g**) S2 feeder cells were added to Matrigel matrix coated wells of 8-well chamber slides. On day 15 post initiation of the culture, oocysts were assessed by IFA using an anti-PfCSP mAb. The culture including S2 cells (**f**) had many oocysts and iPfSPZ, indicated by arrows. The culture without S2 cells (**g**) had significantly lower numbers of oocysts (Supplementary Table 2). The oocysts in the cultures without S2 cells (**g**) had many budding PfSPZ expressing PfCSP (dots), but no fully developed iPfSPZ, suggesting an arrest in development. This experiment was performed three times and photomicrographs taken once.



Extended Data Fig. 2 | Pf life cycle without mosquitoes or humans. iPfSPZ were injected into mice containing humanized livers (FRGhuHep mice). Six and 7 days later the mice were transfused with human erythrocytes, and 6 h after the day 7 transfusion, blood was removed from the mice and cultured *in vitro* using standard methods. **a**, Parasites developed in these cultures to form Pf rings, trophozoites and schizonts; **b**. After induction, Pf gametocytes were produced in the cultures.



Extended Data Fig. 3 | Gene and protein expression of iPfSPZ. a. Scatter plot showing correlation of transcriptomes; x axis mPfSPZ, y axis iPfSPZ, right upper corner in red print, expressed liver stage genes. **b. Heatmap of genes expressed in PfSPZ detected by RNASeq**. 3,656 genes with ≥5 TPM in at least one sample are plotted. The raw gene expression reads from both iPfSPZ and mPfSPZ samples are in Supplementary Table 4. **c. Comparative transcriptomics of mPfSPZ and iPfSPZ**. Volcano plot of differentially expressed genes are plotted based upon average fold change and unadjusted average p-value from the 5 DE tools (significance of differential expression, see methods). Extreme low p-values are cropped at 1e-08 for plotting. Notable, differentially expressed genes are highlighted in red. **d. PfCSP expression by mPfSPZ and iPfSPZ by immunoblot analysis**. Lanes 1-3 are $2.0x10^5$, $5.0x10^4$ and $1.25x10^4$ mPfSPZ, and lanes 5-7 are $1.1x10^6$, $2.0x10^5$, $5.0x10^4$ iPfSPZ respectively. No sample was loaded in lane 4. Molecular size markers are indicated on the left.



Extended Data Fig. 4 | See next page for caption.

Extended Data Fig. 4 | T-cell responses in fresh and cryopreserved splenocytes. Data show spot forming cells (SFC) expressing interferon gamma (IFNγ)/10^6 splenocytes from 6 mice immunized with iPfSPZ and 6 mice immunized with mPfSPZ at 2 weeks after the fourth dose of iPfSPZ or mPfSPZ. Splenocytes from 2 mice were pooled so that there were 3 samples from each group of immunized mice (a, b, c, d, e, f and h). Each pooled splenocyte sample was assayed in duplicate and each data point represents the mean of the duplicates. Results are median and interquartile ranges of SFC/10^6 splenocytes after incubation with stimulating cells at different concentrations. P values were calculated using the Mann-Whitney U test. The same symbol was used for each pool sample at the 3 different concentrations of stimulating cells. **T-cell responses against *Plasmodium falciparum*-infected red blood cells (PfRBC) in fresh (a) and cryopreserved (b) splenocytes from immunized mice**. One pool (●) had no detectable signal at 1x10^5 PfRBC but had a signal at both 2x10^5 PfRBC and 5x10^4 PfRBC. The negative response at 5x10^4 was considered a technical error and removed from the analysis. **c, d. T-cell responses against mPfSPZ in fresh (c) and cryopreserved (d) splenocytes from immunized mice**. Splenocytes were stimulated with 2.5x10^4 mPfSPZ. The responses to incubation with vaccine diluent alone are also shown. **e, f. T-cell responses against uninfected red blood cell (uRBC) in fresh (E) and cryopreserved (F) splenocytes from immunized mice**. The same symbol was used for each pool at the 3 different concentrations of uRBC (2x10^5, 1x10^5, and 5x10^4). **g. T-cell responses against mPfSPZ in fresh splenocytes from naïve mice**. Splenocytes of 2 naïve mice were pooled and frozen before assaying in duplicates and splenocytes of 2 naïve mice were pooled. and assayed fresh in duplicate. Splenocytes from naïve mice were stimulated with 2.5x10^4 mPfSPZ. **h. T-cell responses against PfRBC in fresh and cryopreserved splenocytes from naïve mice**. Fresh and cryopreserved splenocytes from naïve mice were stimulated with 2x10^5, 1x10^5, and 5x10^4 PfRBCs.

Extended Data Table 1 | Transformation efficiency of stage V gametocytes to PfSPZ in mosquitoes and *in vitro* culture

	Mosquitoes (*in vivo*)	*In vitro*			
		Matrigel	Rat Collagen	GMP Collagen	No Matrix
Mean (± SD) number of gametocytes fed to a mosquito or added per well	21,781 ± 3,581[a]	10,000	10,000	10,000	10,000
Mean (±SD) mPfSPZ/mosquito or iPfSPZ/well	70,095 ± 26,687[b]	238,000	139,000	135,000	91,300
Mean mPfSPZ or iPfSPZ/ gametocyte	3.2	23.8	13.9	13.5	9.1

Footnote: iPfSPZ yields were 7.4, 4.3, 4.2 and 2.8-fold more in *in vitro* cultures with Matrigel, rat tail collagen, GMP collagen and no-matrix respectively compared to PfSPZ yield in mosquitoes.
[a]Estimated gametocytes ingested per mosquito (see Extended Data Table 2 footnote).
[b]Calculated based on the average PfSPZ yield in 20 experiments in which mPfSPZ were produced.

Extended Data Table 2 | Transformation efficiency of gametocytes to 8-day oocysts in *in vitro* was 39-fold more as compared to *in vivo* growth and development in mosquitoes

Exp #	Stage V gametocytes /well	Geometric mean of 3-day oocysts/well (range)	Transformation efficiency of gametocytes to 3-day oocysts	Geometric mean of oocysts/well on day 8 (range)	Transformation efficiency of gametocytes to 8-day oocysts	Mean transformation efficiency to 8-day oocysts
1	15,000	428 (180-540)	2.9%	359 (260-375)	2.4%	
2	15,000	2113 (1790-2695)	14.1%	1877 (1470-2585)	12.5%	8.9%
3	15,000	2334 (2235-2405)	15.5%	1786 (1450-2220)	11.9%	
1	25,000	1107 (650-1685)	4.4%	978 (900-1050)	3.9%	
2	25,000	2916 (1635-4515)	11.8%	2210 (1970-2825)	8.8%	8.4%
3	25,000	3888 (3730-4070)	15.5%	3092 (2885-3365)	12.4%	
[a]*In vivo*	[b]21,781 ± 3,581	ND	ND	[c]47.2 ± 32.9[c]	ND	0.22%

Footnote: Numbers of oocysts on days 3 or 8 developing *in vitro* in 8-well chamber slides with Matrigel matrix were assessed by IFA using anti-Pfs25 and anti-PfCSP mAbs respectively. Oocysts from triplicate wells were counted and the geometric mean oocysts/well calculated. Transformation efficiency was the percentages of stage V gametocytes that developed into oocysts. For each experiment the same gametocyte culture was seeded into each well. Stage V gametocyte conversion to 7- or 8-day oocysts in mosquitoes *in vivo* was determined by mercurochrome staining of the mosquito midguts and enumerating the number of oocysts per midgut.

[a]*In vivo* in mosquitoes. [b]Number of gametocytes ingested by a mosquito were estimated based on the mean volume of blood ingested by mosquitoes (N=20) and the estimated stage V gametocytemia of the infectious blood meal. [c]Mean oocysts per mosquito midgut on days 7 or 8 after feeding was the mean of the geometric mean of 74 independent standard membrane feeding assays with 20-25 mosquitoes assessed in each experiment.

Extended Data Table 3 | iPfSPZ produced in 12-well culture plates with S2 cells and no matrix

Experiment number	Number of wells	Total iPfSPZ ($x10^6$)		Purification yield (%)	iPfSPZ/well
		Harvested	Partially purified		
1	72	6.19	1.75	28.3	85,972
2	36	2.63	0.40	15.2	73,056
3	72	7.38	0.95	12.9	102,500
4	72	3.83	0.68	17.6	53,194
5	18	0.95	0.13	13.2	52,778
6	81	1.69	0.18	10.4	20,864
7	84	5.05	1.03	20.4	60,119
8	85	7.34	2.36	32.2	86,353
9	86	2.42	0.68	27.9	28,140
10	144	7.31	0.81	11.1	50,764
11	180	5.58	1.46	26.2	31,000

Footnote: 10,000 stage V gametocytes were seeded per well. iPfSPZ harvested were partially purified and quantitation of iPfSPZ was performed by phase contrast microscopy (x400) using a Cellometer counting chamber. A total of 50.37 x 10^6 PfSPZ were produced in 11 experiments.

Extended Data Table 4 | Infectivity in HC-04 cells of Matrigel matrix-produced iPfSPZ compared to mPfSPZ

Exp. #	Number of iPfSPZ/well		Mean number 6-day parasites/well ± SD		Mean number 6-day parasites/well/ 50,000 PfSPZ ± SD		
	15-day	**18-day**	**15-day iPfSPZ**	**18-day iPfSPZ**	**15-day iPfSPZ**	**18-day iPfSPZ**	**mPfSPZ**
1	60,937	71,500	24.5 ± 6.8	32.3 ± 5.6	20.1	22.6	20.7 ± 3.5
2	47,500	ND	21.7 ± 2.1	ND	22.8	ND	32.7 ± 1.5
3	50,000	55,000	17.0 ± 2.3	37.0 ± 5.0	17.0	33.6	21.3 ± 4.0
4	55,000	56,250	32.3 ± 3.8	35.3 ± 0.9	29.4	31.4	28.3 ± 1.5
Mean ± SD for all 4 experiments					**22.3 ± 5.3**	**29.2 ± 5.8**	**25.7 ± 5.8**

Footnote: iPfSPZ were harvested on days 15 or 18 post culture initiation. The numbers of morphologically mature iPfSPZ harvested were counted using a Cellometer counting chamber. The infectivity of the PfSPZ was determined by counting the numbers of PfMSP-expressing parasites identified by IFA after incubation of the PfSPZ in triplicate wells in cultures of HC-04 cells for 6 days. The numbers of parasites per 50,000 iPfSPZ added was calculated based on the numbers of iPfSPZ actually added to each well. The infectivity of aseptic, purified, cryopreserved mPfSPZ ($5x10^4$ PfSPZ/well) was compared in each experiment. ND, not determined.

Extended Data Table 5 | Infectivity to HC-04 cells of rat tail collagen 1 matrix-produced iPfSPZ compared to mPfSPZ

Number of iPfSPZ seeded/well	Mean number 6-day parasites/well ± SD		
	iPfSPZ	Per 50,000 iPfSPZ	mPfSPZ
38,750	27.0 ± 10.4	34.8	31.0 ± 3.5
24,375	22.0 ± 5.3	45.1	31.0 ± 3.6

Footnote: iPfSPZ were harvested on day 15 post culture initiation. Numbers of morphologically mature iPfSPZ harvested were counted using a Cellometer counting chamber. The infectivity of the PfSPZ was determined by counting the numbers of PfMSP-expressing parasites identified by IFA in a 6-day hepatocyte potency assay in HC-04 cells. Six-day parasite numbers per $5x10^4$ iPfSPZ were calculated by extrapolation. Aseptic, purified, cryopreserved mPfSPZ were seeded at $5x10^4$ PfSPZ/well.

Extended Data Table 6 | Pf life cycle from gametocytes to gametocytes without the use of mosquitoes or primates

Mosquito container number	1	2	3
Number of mosquitoes assessed for oocysts	25	24	22
Oocyst infection prevalence (%)	24	71	82
Oocyst infection intensity	0.2	2.1	4.2
Range of infection	0-4	0-16	0-28
PfSPZ per mosquito	2,144	11,340	24,878

Footnote: Pf gametocytes produced from erythrocytic stage parasites obtained from the first FRGhuHEP mouse infection were fed to *A. stephensi* mosquitoes. Three mosquito containers each with ~100 mosquitoes were fed blood meals containing stage V gametocytes. Infection prevalence (percent of mosquitoes with oocysts) and intensity (geometric mean number of oocysts per mosquito) were determined on day 7 post feeding. The numbers of PfSPZ per mosquito were determined on day 15 post feeding. Salivary glands from 40 mosquitoes from each container were dissected, pooled, extracted and the number PfSPZ per mosquito was determined.

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Reporting Summary

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For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

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☐	☒	The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
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☐	☒	A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒	For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐	For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☐	☒	Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	bcl2fastq2 (version 2.20, Illumina) Bowtie2 Alignment tool (version 2.3.4.2)
Data analysis	R package DuffyNGS (version 1.9.1, https://github.com/robertdouglasmorrison/DuffyNGS) Round Robin [in-house] Rank Product58 Significance Analysis of Microarrays (SAM)59 (R package siggenes, version 1.58.0) EdgeR60 (version 3.26.8); 5) DESeq261 (version 1.24.0)

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Data and Software Availability
The accession number for the RNA-seq fastq files generated in this study is NCBI: PRJNA753927 (https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA753927). To understand the files, please refer to Supplementary Table S3.

PlasmoDB, v.32, https://plasmodb.org/plasmo/app/downloads/release-32/Pfalciparum3D7/
BDGP6.32, https://nov2020.archive.ensembl.org/Drosophila_melanogaster/Info/Index)
A. stephensi (UC Irvine; v1.0,https://www.ncbi.nlm.nih.gov/genome/2653?genome_assembly_id=985930)]

Field-specific reporting

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	With few exceptions, sample sizes were based upon our long (>19 years) experience working with the GMP-based and related assays for PfSPZ. For example, liver stage assays follow a standard operating procedure that has been unchanged for many years and has strict conditions under which an assay is considered acceptable. In a few cases, e.g. infections of FRG-huHep mice, additional constraints were external (e.g. desire to use minimum numbers of animals) and required minimum positive observational data. Sample sizes for the mouse immunogenicity study were based on our long experience with such studies, especially initial, single, preliminary studies.
Data exclusions	No data were excluded.
Replication	Number of replicate assays or infection studies are detailed in the manuscript
Randomization	For mouse studies, mice were randomly allocated to treatment groups. For laboratory studies, randomization was not necessary for the experiments described in this paper because different experimental treatments were being compared against GMP standard material (live, infectious, purified, cryopreserved PfSPZ derived from mosquitoes under strict conditions of asepticity). Randomization was always performed when necessary and possible.
Blinding	Investigators were typically blinded in experiments. This is the norm for readout of, for example, liver assays where the assay reader is not aware of the experimental conditions that were used to derive the PfSPZ that seeded into the hepatocytes. In addition, the assessment of iPfSPZ numbers or mouse infections from cultures was usually performed by a team different to those generating the cultures themselves. For mouse immunogenicity studies, the assay technicians were blinded to mouse treatment.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☐	☒ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data
☒	☐ Dual use research of concern

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used	Alexa Fluor 488 anti-mouse IgG, Invitrogen, Catalog# A32723, RRID# AB_2633275

Antibodies used	Monoclonal antibody (mAb) against 25 kD Pf sexual stage protein, Pfs25 (BEI resources catalogue number MRA-315, clone: Pfs25mAb-4B7); Barr, P. J., et al. "Recombinant Pfs25 Protein of Plasmodium falciparum Elicits Malaria Transmission-Blocking Immunity in Experimental Animals." J. Exp. Med. 174 (1991): 1203-1208. PubMed: 1940798. mAb against Pf circumsporozoite protein (PfCSP) (clone A210, BEI resources catalogue No. MRA-183). mAb against the Pf merozoite surface protein 1 (clone: AD223). mAb against the Pf 70 kD heat shock protein (clone;:4C9). mAb against Pf exported protein 1 (PfEXP1) was made by Sanaria. Rabbit polyclonal antisera to PfLSA1 [Guerin-Marchand, C., et al. A liver-stage-specific antigen of Plasmodium falciparum characterized by gene cloning. Nature 329, 164-167 (1987)]
Validation	All of the antibodies were acquired from BEI resources (Anti-Pfs25 mAb, anti-PfCSP mAb); NIAID, NIH (anti-PfMSP1 mAb); or Johns Hopkins School of Public Health (anti-HSP70 mAb); or were made by Sanaria or one of its contractors by immunizing with purified recombinant protein produced by Sanaria's sister company, Protein Potential LLC (anti-PfEXP1 mAb and anti-PfLSA1 anti-sera). All have been demonstrated by Sanaria to recognize P. falciparum life cycle stages they are expected to recognize and in the case of the anti-PfCSP, anti-PfMSP1, anti-PfEXP1, and anti-PfLSA1 antibodies, recombinant proteins of these antigens.

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)	Spodoptera frugiperda, Sf21 cells (Catalog # 11497013) were purchased from ThermoFisher (Manufacturer's address: Life Technologies Corporation | 5781 Van Allen Way | Carlsbad, CA 92008). These cells are patented and covered under a use license by Thermo Fisher. Drosophila melanogaster Schneider S2 cells (Catalog # R69007) were purchased from ThermoFisher (Manufacturer's address: Life Technologies Corporation | 5781 Van Allen Way | Carlsbad, CA 92008). These cells are patented and covered under a use license by Thermo Fisher.
Authentication	At ATCC the HC04 cell line has been tested for genetic profiles, STR (Short-tandem-repeats) and HLA-class I molecules. Karyotypic analysis performed by the contributor indicated that the cells are all in the hyperdiploid range (2n = 48-50).1 Abnormal chromosomes include a chromosome 1p deletion, a chromosome 6 derivative, triplet of chromosome 7, and a chromosome 15 derivative. STR analysis from both passage 8 and passage 56 cells provided by two independent laboratories at the WRAIR in 2009-2010 produced identical STR results shown below (identical results were also obtained by BEI Resources). Amelogenin X,Y CSF1PO 10,11 D13S317 9,13 D16S539 12 D5S818 11,12 D7S820 10 THO1 9 TPOX 8,9 Vwa 17 S2 cells and Sf21 were purchased from Thermo Fischer. The CoA authenticates the cells. Purchased cells were used to make a cell bank. Vials are thawed and the cells were used until passage 10.
Mycoplasma contamination	S2 and Sf21 cells are provided with a certificate of analysis indicating they are free of Mycoplasma. Sanaria's bank of HC-04 cells was tested by an outside contractor and determined to be Mycoplasma free.
Commonly misidentified lines (See ICLAC register)	No commonly misidentified cell lines was used in the study.

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals	Female 6-8 weeks FRG-KO huHep mice (on a NOD background) with human chimeric livers were purchased from Yecuris Corp (P.O. Box 4645, Tualatin, OR 97062). All mice had human liver repopulation levels above 70%, and established protocols for injecting the mice with Pf sporozoites were followed. Six to eight-week-old BALB/c were housed at Bioqual, Maryland, and all protocols were approved by the Bioqual institutional IACUC
Wild animals	No wild animals were used in this study.
Field-collected samples	No field collected samples were used in this study.
Ethics oversight	The Institutional Animal Care and Use Committees (IACUC) reviewed and approved all procedures and monitoring prior to the experiment being conducted. Experiments were performed in accordance with relevant guidelines and regulations.

Note that full information on the approval of the study protocol must also be provided in the manuscript.